Supplementary material

Materials and methods:

DNA constructs

Biotinylated and/or amine-modified 30-base and 43-base DNA oligonucleotides were purchased, PAGE-purified, from Qiagen. Amine-modified oligos were labeled with amine-reactive Cy3 or Cy5 (Amersham Bioscience) post-synthetically following the protocol provided by the supplier. The dye-labeled oligos were purified using reverse phase HPLC. Complementary strands of DNA were annealed to form biotinylated double-stranded DNA (dsDNA) constructs that contained either a single Cy5 or both Cy3 and Cy5. Annealing was carried out by mixing equimolar amounts of the two complementary strands in 10 mM Tris-Cl (pH 7.5), 50 mM NaCl, heating to 90 degrees C for two minutes, and then cooling the mixture to room temperature in a heat block over a period of one hour.

The following DNA oligos were used to form the dsDNA constructs. Note that [AmC7-Q] and [AmC12] indicate an end-positioned amine modification with a 7-carbon or a 12-carbon linker, respectively, [AmC6-dT] indicates an internal dT modified with an amine group connected via a 6-carbon linker, and [BioTEG-Q] indicates a biotin modifier. DNA sequences are listed from 5' to 3'. The location of the amine modified dT specifies the attachment point of the dye to the DNA.

SDA:
CTGCTTCGCGATGTACGTGCCGGATATACG[AmC7-Q]

SDB:
CGTATATCCGGCACGTACATCGCGAAGCAG[BioTEG-Q]

SDC:
[AmC12]CGTATATCCGGCACGTACATCGCGAAGCAG[BioTEG-Q]

RXA:
GCGT[AmC6-dT]ATTACGATCAGTATCATTACCATTGCATACGTTAGCAT[BioTEG-Q]

RXA_B1:
GCGT[AmC6-dT]ATTACAATCAGTATCATTACCATTGCATACGTTAGCAT[BioTEG-Q]

RYA:
ATGCTAACGTATGCAATGGTAATGATACTGATCGTAA[AmC6-dT]AACGC

RYB:
ATGCTAACGTATGCAATGGTAATGATACTGATCG[AmC6-dT]AATAACGC

RYC:
ATGCTAACGTATGCAATGGTAATGATACTGA[AmC6-dT]CGTAATAACGC

RYD:
ATGCTAACGTATGCAATGGTAATGATAC[AmC6-dT]GATCGTAATAACGC

RYE:
ATGCTAACGTATGCAATGGTAATGA[AmC6-dT]ACTGATCGTAATAACGC

Experimental data presented in figures 1, 2, and 3 were obtained using Cy3-labeled SDC annealed to Cy5-labeled SDA. The control experiment in which no Cy3 was present was performed using unlabeled SDB annealed to Cy5-labeled SDA. Data presented in figure 4 were obtained using Cy3-labeled RXA annealed to one of Cy5-labeled RYA, Cy5-labeled RYB, Cy5-labeled RYC, Cy5-labeled RYD, or Cy5-labeled RYE. The control experiment in which there was a one base-pair mismatch in the dsDNA structure was performed using Cy3-labeled RXA_B1 annealed to Cy5-labeled RYD.

The intermolecular distance *R* between the attachment sites of the Cy3 and Cy5 dyes in the dsDNA constructs used in figure 4 was calculated from the crystal structure of standard B-form DNA using Swiss PDB Viewer and the sequences above. In these constructs, the dye linker is attached at carbon 5 of the pyrimidine ring of specific thymine bases (as indicated above) and this atom is referred to as the dye "attachment site".

To facilitate single-molecule detection, the DNA constructs were immobilized via a streptavidin-biotin linkage to either biotin-BSA- or biotin-PEG-coated fused quartz slides. Biotin-BSA slides were prepared by incubating a quartz slide with biotinylated BSA solution. The slides were then rinsed with buffer (10 mM Tris, 10 mM NaCl), incubated with streptavidin, rinsed again with buffer, and incubated with the annealed DNA solution to immobilize the DNA to the slide. To prepare biotin-PEG slides, quartz slides were treated with an amino-silane reagent, Vectabond (Vector), and then incubated with a 20% (w/v) PEG:biotin-PEG (50:1) solution in 0.1 M sodium bicarbonate buffer (pH 8.3) for 3 h. Slides were rinsed with dH_2O and dried with N_2 before a streptavidin solution was applied. Biotinylated DNA samples were then immoblized to the surface in a similar fashion to the biotin-BSA slides [Ha et al., Nature **419**, 638 (2002)].

The hairpin ribozyme

The four strands that form the hairpin ribozyme (4-way junction) were gifts from Dr. Nils Walter, University of Michigan (Ann Arbor, MI). The RNA strands were synthesized by the Howard Hughes Medical Institute Biopolymer/Keck Foundation Biotechnology Resource Laboratory (Yale University). Cy5 was attached to the RzA strand during synthesis. Cy3 was coupled to the 3' end of RzA post-synthetically, via a primary amine attached to the 3'-most phosphate. RNA strands were gel purified and reverse phase HPLC purified. The strand sequences (5' to 3') are shown below. The wildtype 4-way junction has a self-cleaving activity which is removed in our construct. The SV4WmA-1 strand was modified with a 2'-methoxy group on the adenine at the cleavage site (indicated by "mA" below) such that it becomes non-cleavable, but retains the docking and undocking properties [Walter et al., Nat. Struct. Biol. **6**, 544 (1999)].

RzA:
[Cy5]-AAA UAG AGA AGC GAA CCA GAG AAA CAC ACG CCA AA-[Cy3]

BRzB4W:
[Biotin]-AUA UAU UUG GCG UGG UAC AUU ACC UGG UAC GAG UUG AC

RzC4W:
GUC AAC UCG UGG UGG CUU GC

SV4WmA-1:
GCA AGC CAC CUC GCmA GUC CUA UUU

Annealing was carried out by mixing SV4WmA-1:RzC4W:BRzB4W:RzA in a 3:3:1:1 molar ratio in an annealing buffer containing 25 mM Tris-Cl (pH 7.5), 0.5 mM EDTA, 50 mM NaCl, and 1 % (v/v) beta-mercaptoethanol. This mixture was heated to 90 degrees C for two minutes and then cooled to room temperature in a heat block over a period of two hours. For single molecule detection, the annealed RNA molecules were immobilized to biotin-BSA coated surfaces via streptavidin-biotin linkage as described for DNA samples.

Single molecule imaging

An Olympus IX70 microscope was used for single-molecule imaging with the prism-type total-internal-reflection excitation scheme. Samples were excited with a 638 nm diode laser (Crystalaser) and a 532 nm diode-pumped Nd:YAG laser (Crystalaser) or a mercury lamp. The fluorescence emission of Cy5 was collected with a N.A. 1.25 60X water immersion objective, and imaged onto an intensified CCD camera (Pentamax; Roper Scientific) after passing through a 665 nm longpass filter (Chroma). For FRET measurements, the molecular switches were excited with the 532 nm laser. The fluorescence from Cy3 and Cy5 was filter with a 550 longpass filter (Chroma) and split using a 630 nm dichroic mirror and imaged onto two separate areas of the camera. Images were recorded at a frame rate of 10 Hz.

Labeled dsDNA strands were imaged in Tris buffer (pH 7.5) with a glucose oxidase catalase oxygen scavenging system. Specifically, the DNA imaging buffer contained 10 mM Tris-Cl, 10 mM NaCl with an oxygen scavenger system (10% (w/v) glucose, 1% (v/v) β-mercaptoethanol, 200 μg/mL glucose oxidase, and 10 μg/mL catalase). Sucrose and potassium iodide were added for some experiments, as noted in the text.

The hairpin ribozyme was diluted prior to imaging into a low ionic strength buffer (50 mM Tris (pH 7.5), 10 mM NaCl, 1 mM EDTA,) or a high ionic strength buffer (50 mM Tris (pH 7.5), 100 mM NaCl, 10 mM $MgCl_2$). In both cases, the same oxygen scavenger system was used as in the DNA samples.

Excitation of Cy5 alone at different wavelengths

We tested broad-spectrum light generated by a mercury lamp (Olympus U-LH100L-3) for its ability to cause Cy5 to revert to the fluorescent state in the absence of Cy3. The spectrum of light from the lamp contains peaks at 577 nm, 546nm, 435 nm, 405 nm, 365 nm, with comparable intensity and several other lines in the UV range.

First, we verified that the lamp intensity was sufficient to excite the Cy5 dark state for constructs in which Cy3 was present. The first construct tested was Cy5-SDA annealed to

Cy3-SDC. The Cy5 molecules for a given field of view were first converted to the dark state using the red laser. The red laser was turned off and the sample was exposed to the mercury lamp excitation for 5 seconds. The red laser was turned back on and it was observed that the 5 second pulse of light from the mercury lamp was sufficient to convert most of the Cy5 molecules back to the fluorescent state. Next, a sample consisting of Cy5-SDA annealed to SDB was tested. This sample lacks a Cy3 dye molecule. Following the same procedure as for the previous sample, we did not observe any recovery of Cy5.

Supplementary video:

This movie shows the photo-induced reversible switching of single Cy5 molecules between the dark and fluorescent states, facilitated by the presence of a Cy3 molecule. In the movie, the red laser continuously excites the sample. The green laser is periodically turned on and off, effectively "gating" the fluorescence output of the Cy5. Time-dependent red and green excitation is indicated by the red and green squares at the lower right and lower left corners of the video, respectively. Red-orange spots on a dark blue background indicate fluorescence signals from individual Cy5 molecules. The sample used for this movie was Cy5-SDA annealed to Cy3-SDC. The movie was compressed with Quicktime Pro and runs at 3× real time.

Supplementary Figure:

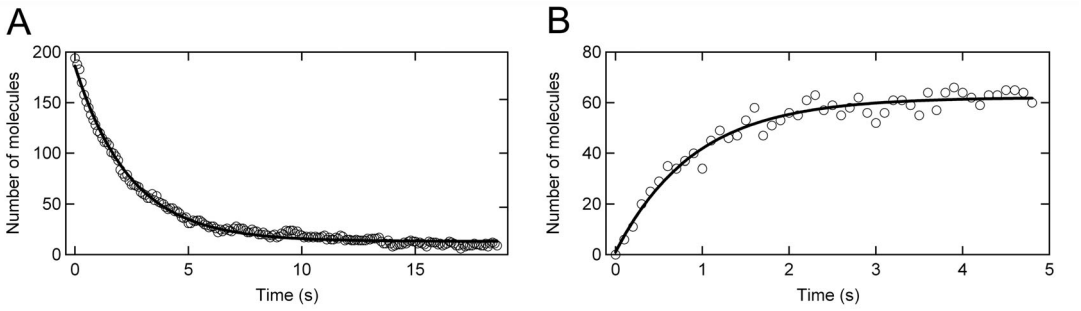

Figure 1. The first-order switching kinetics of the molecular switch. **A**, The number of molecules remaining fluorescent as a function of time after the green laser was turned off. A single exponential fit of the data (solid line) gives $k_{off} = 0.4\ s^{-1}$. **B**, The number of molecules that were converted back to the fluorescent state as a function of time after the green laser was turned on. A single exponential fit (solid line) gives the observed rate constant for switching Cy5 on ($k_{on\_obs} = 1.1\ s^{-1}$). Considering the competing actions of the red and green lasers, the actual rate constant k_{on} for switching the dye on by the green laser is equal to $k_{on\_obs} - k_{off}$. Data in **A** and **B** are not from the same experiment.